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ESTIMATION OF SELFING RATE IN A NATURAL POPULATION OF SCROPHULARIA NODOSA L. USING ALLOZYMES

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SUMMARY

The genetics of 4 polymorphic enzyme systems PO, 6PGDH, PGM1 and PGM2, is investigated in a seed population derived from a natural population of *Scrophularia nodosa*. No linkage was detected between any of the four loci. Two of the loci are used to estimate outcrossing rates in a natural population by single-locus as well as multilocus methods. The absence of selfing is in contradiction with the expectations. Testing the fitness of alien pollen against self pollen by performing artificial crosses with different pollen mixtures with PO as a genetic marker revealed no differences between alien and own pollen. Therefore the mechanism preventing self-pollination in this natural population of *S. nodosa* remains unresolved. The ecological significance of the observed mating system is discussed.

1. INTRODUCTION

In investigating the genetical variation in natural plant populations and its maintenance, the exact determination of the mating system is of great importance (JAIN 1975, ALLARD 1975, RICK 1976, CLEGG 1980). In plant species, breeding systems can vary widely, from cleistogamy up to obligate xenogamy, resulting in predominant inbreeding or outbreeding respectively (JAIN 1976). Different ways of mating result in a different composition of the gene pool starting the next generation, and thus are of great consequence for the conservation, loss or gain of genetical variation, and therefore for evolution.

Observations of pollinator behaviour and flower morphology have yielded valuable, though fundamentally qualitative insights into the mating process in plants (CLEGG 1980). However, only well defined genetic markers permit valid estimations of the actual result of the observed pollinations.

Using codominant allozyme markers, it is possible to estimate outcrossing rates from genotypic arrays of half-sib families in which the maternal plant is of unknown genotype; in these estimates the genotype of the maternal plant is inferred from the array of progeny produced by each maternal individual (BROWN & ALLARD 1970, CLEGG et al. 1978, BROWN et al. 1975). Comparison of single locus and multilocus estimation methods can distinguish selfing from other forms of inbreeding (SHAW & ALLARD 1979).

In this study we estimate the selfing rate in a natural population of *Scrophularia nodosa*, a perennial geophyte, occurring on woodland clearings. Two polymorphic enzyme systems are used in the estimation procedure. Observations of pollinator behaviour, glasshouse experiments and flower morphology suggested that selfing might occur to a certain extent in natural populations of *S. nodosa* (VAN BAALEN 1982). Especially geitonogamy (i.e. pollination between flowers of the same plant, see JAIN 1976) could play an important role. Although *S. nodosa* is slightly protogynous, flowers of all stages are distributed more or less randomly all over the plant, and foraging behaviour of potential pollinators (wasps, bees and small bumblebees) was not observed to occur in a way that would prevent selfpollination.

In the glasshouse *S. hodosa* seemed fully self-compatible in our crossing experiments; seed production, germination and growth rate did not differ distinctly between selfed and outcrossed progeny (see also VAN BAALEN 1982).

The population examined in this study was growing on the acidic part of a valley slope near Orsbach (GDR; 6°.02′E, 50°.47′N), and consisted of over 500 individuals (see DE NEELING 1982). The population was 4 years old at the time of sampling, and most of the individuals were in their second season (VAN BAALEN 1982).

Investigations on genetical variation revealed 3 polymorphic enzyme systems out of 24 examined.

In this paper we also present genetic studies, including linkage testing with these enzyme systems. Although the chance that linkage is involved is very small, it has to be excluded, because independent segregation is one of the assumptions in the multilocus estimation method (SHAW et al. 1981). Following the estimation of inbreeding and selfing rates, an attempt is made to explain the results experimentally, using PO genotypes as a genetic marker.

2. MATERIAL AND METHODS

2.1. Electrophoresis and staining techniques

Electrophoresis and staining techniques are described elsewhere (KONIUSZEK & VERKLEIJ 1982, VERKLEIJ & KONIUSZEK, 1981).

2.2. Genetics and linkage of polymorphic enzymes

Parents of known phenotypes for PO, 6PGDH and PGM were artificially crossed in the glasshouse in order to detect the genetic basis of the enzyme polymorphism.

Parental plants with different genotypes combinations for these enzymes, were used to examine the linkage relationships between PO and 6PGDH, between 6PGDH and PGM1, between PO and PGM1, and between PGM1 and PGM2. The observed gene segregation at each locus was used to calculate the expected number for each of the two-locus segregants (BAILEY 1961) as:

P-generation		ss1ss2 × sf1sf2		
F_1	ss1ss2	ss1sf2	sf1ss2	sf1sf2
expected frequencies	$\frac{a1a2}{n^2}$	$\frac{a1b2}{n^2}$	$\frac{b1a2}{n^2}$	$\frac{b1b2}{n^2}$

as: n = total number of offspring
a1 = number of ss1-offspring
a2 = number of ss2-offspring
b1 = number of sf1-offspring
b2 = number of sf2-offspring

Chi-square tests for goodness of fit were used to test deviations from expected frequencies; in case of an expected number smaller than 5, found and expected numbers of these classes are pooled in order to obtain a joint class with an expected number larger than 5 (SOKAL & ROHLF 1969).

2.3. Estimates of outcrossing rates in natural populations

2.3.1. Sampling method

From 60 randomly chosen individual plants in a large population (over 500 individuals) of *S. nodosa*, seeds were obtained in the late summer of 1981 before falling of the seeds had started.

In the glasshouse these seeds were sown in plastic containers in pure calceolaria peat soil (t: 22° C; l/d: 12 h; r.h.: 70%). In an early growth stage (2–4 leaves) 60 plants were randomly chosen from each container (family) and used for electrophoresis to determine PO and 6PGDH genotypes. At the same time a bulk seed sample was examined to obtain an estimation of allele frequencies in the pollen pool (ALLARD et al. 1977).

2.3.2. Single locus estimation procedure

In this method PO and 6PGDH results are used separately in a joint maximum likelihood estimation of outcrossing rate ($\hat{t}$), using the mixed mating model (BROWN et al. 1975). The assumptions of this model are: a) mating is caused by random outcrossing or selfing, b) the pollen pool is homogeneous over the whole population, c) the probability of an outcross is not affected by the maternal genotype and, d) selection does not intervene between mating and the determination of progeny distribution (SHAW & ALLARD 1979).

Heterogeneity of the pollen pool can lead to an overestimation of the amount of selfing caused by the Wahlund effect (WAHLUND 1928). We have no indications from our crossing experiments in the glasshouse with *S. nodosa*, that the assumptions c) and d) are not valid.

2.3.3. Multilocus estimation procedure

In this procedure PO and 6PDGH results are used together according to the method of SHAW et al. (1981). This method gives a better estimation of the selfing

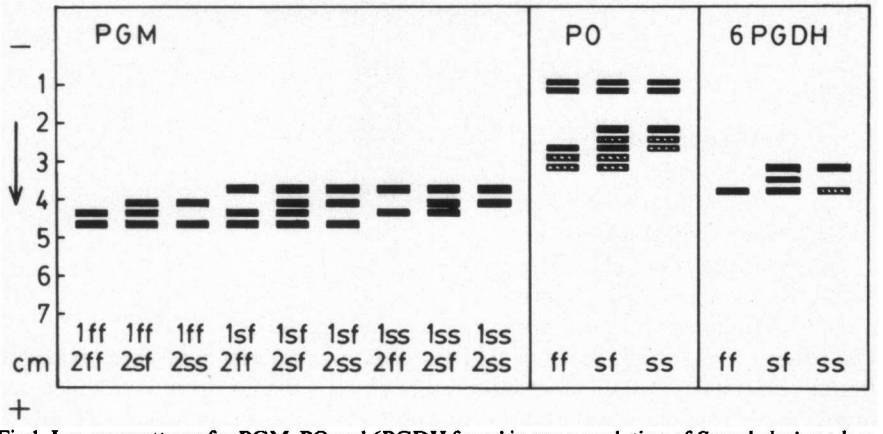

Fig 1. Isozyme patterns for PGM, PO and 6PGDH found in one population of *Scrophularia nodosa*. S and f are alleles respectively coding for slow and fast migrating colored zones on the polyacrylamide gels.

rate in comparison with the single locus method, less biased by the Wahlund effect.

One addditional assumption in this model is that there is no linkage between the loci involved (SHAW et al. 1981, SHAW & ALLARD 1979). Comparison of the results of both estimation methods also gives an indication of inbreeding in natural populations not caused by self fertilization (SHAW & ALLARD 1979).

2.3.4. Testing the significance of the results

Significant deviations from $t = 1$ (i.e. 100% random outcrossing) were tested using the test-statistic

$$\frac{(\hat{t} - t_0)^2}{\mathrm{var}(\hat{t})},$$

which follows (asymptotically) a χ^2-distribution with one degree of freedom (RAO 1973).

2.4. Testing of incompatibility, using pollen mixtures

In this experiment we used different pollen mixtures in pollinating different PO-genotypes of *S. nodosa* artificially in the glasshouse. Seeds that resulted from this experiment were sown and plants were raised and screened for PO-genotype as described above.

3. RESULTS

3.1. Genetics and linkage of polymorphic enzymes in Scrophularia nodosa

Fig. 1 shows the different iso-enzyme patterns for PGM, PO and 6PGDH, found in the population of *S. nodosa* studied here.

Table 1. Numbers of genotypes found and expected for one-locus segregation in offspring from different crosses with known parents for 4 loci in *Scrophularia nodosa*. n = number of replicated crosses.

		offspring					
locus	cross	ss	sf	ff	n	df	χ^2
PO	ss × ss	40(40.0)	–	–	1	–	–
	ss × sf	70(80.0)	90(80.0)	–	4	1	2.50 n.s.
	sf × sf	41(40.0)	78(80.0)	41(40.0)	4	2	0.10 n.s.
	sf × ff	–	36(35.5)	35(35.5)	2	1	0.01 n.s.
	ff × ff	–	–	87(87.0)	2	–	–
6PGDH	ss × ss	105(105.0)	–	–	3	–	–
	ss × sf	110(114.5)	119(114.5)	–	6	1	0.35 n.s.
	sf × sf	33(30.0)	59(60.0)	28(30.0)	3	2	0.45 n.s.
	ss × ff	–	36(36.0)	–	1	–	–
	ff × ff	–	–	26(26.0)	1	–	–
PGM1	ss × ss	94(94.0)	–	–	3	–	–
	ss × sf	117(123.0)	129(123.0)	–	9	1	0.58 n.s.
	sf × sf	17(19.5)	45(39.0)	16(19.5)	3	2	1.87 n.s.
	ff × ff	–	–	120(120.0)	3	–	–
PGM2	sf × sf	33(29.0)	50(58.0)	33(29.0)	6	2	2.21 n.s.
	sf × ff	–	23(31.0)	39(31.0)	4	1	4.13**
	ff × ff	–	–	360(360.0)	9	–	–

** $P < 0.05$

The results from crosses with different phenotypes, given in *table 1*, indicate that the polymorphisms in PO and 6PGDH are due each to one diallellic locus. The banding pattern of PGM is somewhat more complicated, but the crosses indicate a system of two loci with two alleles each.

The result of the ff × sf types from PGM2 does not fully agree with the expectations, but, as this is caused by a deviation in only one out of four replicated crosses with different parents, we must conclude that the diallellic locus system fits with the found frequencies; *Table 2* shows the results from crosses involving various genotypes of the four enzyme genes. These were performed to investigate linkage relationships between the four loci.

Combinations of more than two loci are omitted in this experiment, because the possible number of offspring classes becomes too great for tests of significance based on the present number of offspring.

None of the genotype frequencies shown in *table 2* deviates significantly from expected numbers,, Therefore, no linkage could be detected between the loci for PO, 6PGDH and PGM1, and between PGM1 and PGM2.

3.2. Estimates of outcrossing rates in a natural population

In *table 3* we see the genotype distribution of the polymorphic loci as found in the seed sample of the population under study. The results show a significant

Table 2. Numbers of genotype combinations found and expected under the assumption of random segregation in offspring from crosses with known parents, in order to test linkage relationships. n = number of individuals tested; a: classes pooled in order to obtain a joint class with an expected number larger than 5 (see text).

cross combination	ss1ss2	ss1sf2	ss1ff2	sf1ss2	sf1sf2	sf1ff2	ff1ss2	ff1sf2	ff1ff2	n	df	χ^2
PO(1)–6PGDH(2)												
ss1sf2 × sf1ss2	30(24.6)	19(25.0)	–	29(34.4)	41(35.0)	–	–	–	–	119	3	4.50 n.s.
ss1sf2 × sf1sf2	8(4.5)[a]	9(9.5)	3(6.0)	1(4.5)[a]	10(9.5)	9(6.0)	–	–	–	40	4	3.05 n.s.
ff1ss2 × sf1sf2	–	–	–	9(9.0)	11(11.0)	–	8(8.1)	10(9.9)	–	38	3	0.00 n.s.
sf1ss2 × sf1sf2	6(6.5)	7(6.5)	–	9(8.5)	8(8.5)	–	5(5.0)	5(5.0)	–	40	5	0.14 n.s.
sf1sf2 × sf1sf2	3(3.6)[a]	9(7.8)	1(1.6)[a]	4(5.0)	11(10.8)	3(2.2)[a]	4(2.5)[a]	4(5.4)	1(1.1)[a]	40	4	0.84 n.s.
PO(1)–PGM1(2)												
ss1sf2 × sf1ss2	20(21.2)	30(28.8)	–	31(29.8)	39(40.2)	–	–	–	–	120	3	0.20 n.s.
ss1sf2 × sf1sf2	2(2.5)[a]	14(12.0)	4(5.5)	3(2.5)[a]	10(12.0)	7(5.5)	–	–	–	40	4	1.48 n.s.
sf1ss2 × sf1sf2	7(6.5)	6(6.5)	–	8(8.5)	9(8.5)	–	5(5.0)	5(5.0)	–	40	5	0.14 n.s.
6PGDH(1)–PGM1(2)												
sf1sf2 × ss1ss2	30(35.1)	49(43.9)	–	40(35.5)	40(44.5)	–	–	–	–	159	3	2.36 n.s.
sf1sf2 × sf1sf2	2(1.1)[a]	5(5.4)	2(2.5)[a]	1(2.4)[a]	12(11.4)	6(5.2)	2(1.5)[a]	7(7.2)	3(3.3)[a]	40	4	0.25 n.s.
PGM1(1)–PGM1(2)												
sf1sf2 × ss1sf2	4(7.2)	21(19.1)	5(3.6)[a]	10(6.8)	16(17.9)	2(3.4)[a]	–	–	–	58	4	3.32 n.s.
sf1sf2 × sf1sf2	4(5.7)	5(3.8)[a]	3(2.5)[a]	11(9.9)	5(6.6)	5(4.4)[a]	3(2.4)[a]	2(1.6)[a]	0(1.1)[a]	38	3	1.32 n.s.
sf1sf2 × ss1ff2	–	6(6.7)	8(7.3)	–	5(4.3)[a]	4(4.7)[a]	–	–	–	23	2	0.14 n.s.

Table 3. Numbers of genotypes found and expected on the basis of Hardy-Weinberg equilibrium (brackets) for 4 loci in one population of *Scrophularia nodosa*. n = number of individuals tested; classes pooled in order to obtain a joint class with an expected number larger than 5 (see text).

	genotype				
locus	ss	sf	ff	n	χ^2
PO	32(26.2)	48(59.7)	40(34.1)	120	4.60 n.s.
6PGDH	54(48.9)	44(54.1)	20(15.0)	118	4.08 n.s.
PGM1	106(99.2)	40(53.6)	14(7.2)	160	10.34***
PGM2	0(0.1)[a]	9(8.7)[a]	151(151.2)	160	0.05 n.s.

*** $P < 0.01$

Table 4. Allel frequencies of two loci in the adult population and the pollen pool of one population of *Scrophularia nodosa*, and single-locus and multilocus estimators of outcrossing including standard errors

	alleles		allel f		t (± s.e.)
locus	adult pop.	pollen pool	adult pop.	pollen pool	
6PGDH	.550	.644	.450	.356	.831 (± .051)***
PO	.425	.467	.575	.533	.978 (± .049)***
multilocus estimator t_m (± s.e.)					1.034 (± .048)

number of plants scored in the adult population = 60
number of plants scored in the seed population = 120
*** significantly deviating from 1 ($P < 0.01$)

deviation from Hardy-Weinberg equilibrium in the case of PGM1. This deviation is due to a shortage of heterozygotes in the seed population.

Although the other three loci do not show a significant deviation, we see in the case of 6PGDH and of PO also a shortage of heterozygotes. Pooling of these data provides the following set:

	observed	expected	χ^2
heterozygotes	141	176.1	10.22
homozygotes	417	381.9	

which means, that there exists a significant shortage of heterozygotes in this population, which could be due to inbreeding.

The results of the single locus and multilocus estimations of outcrossing in a natural population of *S. nodosa* are shown in *table 4*. At the time of this investigation, the genetical background of the PGM system was not yet clear, so we did not use the loci involved. We can see, that the estimated outcrossing rate (t) differs depending on the locus that is used: using 6PGDH results in an outcrossing rate of 0.83, significantly deviating from unity, and when we use PO as marker, we find an outcrossing rate of 0.98 which is not deviating from random outcrossing.

Table 5. Number of PO genotypes found and number expected assuming absence of selection (brackets) in offspring resulting from pollination of known parental plants with pollen mixtures

Plant number and offspring PO genotype ♀♀	♂♂	ss	sf	ff	n	df	χ^2
3ff	3ff/56ss	–	31(31.5)	32(31.5)	63	1	0.02 n.s.
56ss	3ff/56ss	43(33.5)	24(33.5)	–	67	1	5.39**xx
37ff	37ff/23ss	–	30(25.0)	20(25.0)	50	1	2.00 n.s.
23ss	37ff/23ss	23(19.5)	16(19.5)	–	39	1	1.26 n.s.
33ff	37ff/23ss	–	17(12.5)	8(12.5)	25	1	3.24 n.s.
2ss	37ff/23ss	13(12.5)	12(12.5)	–	25	1	0.04 n.s.
3ff	33ff/53ss	–	8(7.0)	6(7.0)	14	1	0.29 n.s.
53ss	33ff/53ss	4(6.5)	9(6.5)	–	13	1	1.92 n.s.
21ff	17sf/21ff	–	11(12.5)	39(37.5)	50	1	0.24 n.s.
17sf	17sf/21ff	3(9.4)	41(37.5)	31(28.1)	75	2	4.98 n.s.
33ff	17sf/21ff	–	5(6.3)	20(18.7)	25	1	0.36 n.s.

** $P < 0.05$

The multilocus estimator ($\hat{t}_m$) does not significantly differ from unity, in fact it is 1.03, which means, that no selfing occurs in this population of *S. nodosa*; the fact that the estimate is more than 1, could even indicate that some amount of incompatibility between identical genotypes occurs.

Finding an outcrossing rate smaller than 1 for one locus indicates that some amount of inbreeding, caused by population structure, occurs in this population.

The results of this experiment are in conflict with our expectations. We expected a fair amount of selfing in this population, especially caused by geitonogamy.

To investigate this discrepancy, we investigated if pollen competition on the stigma prevents selfing in this large natural population. In a dense population, pollen of different genotypes will be present on the stigma at the same time, which is clearly not the case in the artificial pollinations in the glasshouse.

Better germination and growth of alien pollen over selfpollen, can prevent selfing in the natural situation.

3.3. Testing of incompatibility, using pollen mixtures

To test whether alien pollen is more efficient than selfpollen when applied to the stigma at the same time, different pollen mixtures were composed and artificially put onto the stigma of different genotypes of *S. nodosa*. As marker we used PO.

In *tabel 5* we see progeny tests of all succesful pollinations; starting from the hypothesis, that no selection takes place after pollination, we tested the results against expected numbers with a chi square test for goodness of fit.

The tests indicate, that, with the exception of one case, there is no significant difference in efficiency between the pollen types. The deviation found in the second case could easily be due to other causes than differences in efficiency, for instance a deviating compostion of the pollen mixture applied to the stigma.

In this experiment we cannot detect any competition between pollen on or in the pistil in *S. nodosa* and therefore we cannot explain the difference between expected and actual outcrossing rates in this population.

4. DISCUSSION

In this paper we investigated inbreeding and selfing rates in a natural population of *Scrophularia nodosa* with the aid of two unlinked polymorphic allozyme systems, PO and 6PGDH.

Using the multilocus estimator $\hat{t}_m$, mating in this population appeared to be random, which means that no selfing was found.

Calculating both single locus estimators $\hat{t}_{PO}$ and $\hat{t}_{6PDGH}$ we see a significant deviation from 1 for $\hat{t}_{6PDGH}$, which means that some degree of inbreeding is found for this locus; $\hat{t}_{PO}$ however, is not significantly deviating from 1. The inbreeding found for 6PDGH is most probably caused by heterogeneity of the pollen pool and the resulting Wahlund effect (WAHLUND 1928). Different outcrossing rates within one population, varying with different loci have already been found in other investigations (ALLARD et al. 1977, HARDING & TUCKER 1964, SHAW & ALLARD 1979). A clear explanation for this phenomenon is not yet given, but it is obvious that the estimates are affected by factors other than the mating system, for instance population structure.

The results of this investigation are quite unexpected, as *S. nodosa* is fully self-compatible in glasshouse experiments and, in view of the spatial structure of the plant, geitonogamy should be occurring on a reasonable scale in natural populations. The foraging behaviour of possible pollinators (wasps, bees and small bumblebees) suggests that this should lead to a fair amount of selfing (VAN BAALEN 1982).

The results forced us to look for some kind of mechanism preventing self-pollination in dense natural populations; in this respect we investigated progeny of different individuals, artificially pollinated with different pollen mixtures; as genetic marker we used PO-genotypes.

In this experiment we found no evidence, that competition between different pollen on the stigma or in the style results in prevention of selfing, as is demonstrated in *Trifolium pratense* (HESLOP-HARRISON & HESLOP-HARRISON 1982).

Perhaps we find an explanation for this discrepancy between the result of natural and artificial pollination in the fact that in a dense population, pollination is always performed as soon as the stigma ripens; in the glasshouse, pollination may be delayed several hours to several days after ripening of the stigma. According to DE NETTANCOURT (1977), it has been shown in a few cases that delayed pollination can break down self-incompatibility barriers and lead to a certain amount of selfing.

Other studies indicate that reproductive methods show great plasticity, and may vary in different environments (FAEGRI & VAN DER PIJL 1966).

However, as a pioneer species on woodland clearings (VAN BAALEN 1982), *S. nodosa* must have the ability for self-fertilization; colonization of new habitats

will be started with very few individuals at a relatively large distance from each other. Clearings are created irregularly in time and space and last only a few years; pioneer species of this habitat are therefore forced to produce numerous progeny in the first one or two years of colonization from a small number of original colonizers. In this period selfing is the obvious strategy to follow (BAKER 1955). This will always lead to a loss of genetic variation and inbreeding depression; in the following stages of succession outcrossing is the most efficient strategy in restoring the genetic variation and preventing extinction of the population due to inbreeding.

We have indications, that *Chamaenerion angustifolium*, another pioneer species on woodland clearings, shows no selfing in large populations, while it is self-compatible in glasshouse experiments, at least in the first generation; further selfing of this generation resulted in severe inbreeding depression (KONIUSZEK, unpublished).

In the case of *S. nodosa* however, we have not investigated yet how inbreeding affects the fitness of the species, but it seems, that we are dealing in both cases with a variable mating system, clearly adapted to the unstable environment, these species have to deal with.

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